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Heterobasidion annosum sensu stricto Pathogenesis: Bioinformatic and Functional Study of Cerato-platanin Family Proteins

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination in Lecture Room B2, B-Building (Latokartanonkaari 7), on November 6th 2015, at 12 noon.

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Cover: Fruiting body of *Heterobasidion annosum s.l.* on Norway spruce root in nature (Eeva Terhonen) (upper left); Saprotrophic growth of *Heterobasidion annosum s.s.* on grounded dead Scots pine sapwood and bark (upper right); Scots pine seedling roots treated with H_2O, BSA, 38 µM HaCPL2, 120 µM HaCPL2 and *H. annosum s.s.* (bottom left); *Nicotiana tabacum* leaves treated with H_2O, vector, BSA, 38 µM HaCPL2, 120 µM HaCPL2 (bottom right).

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To my family and friends

for their love, supports and laughs

CONTENTS

ABBREVIATIONS

cDNA	complementary deoxyribonucleic acid
CWDE	cell wall-degrading enzyme
DAMP	damage-associated molecular pattern
DNA	deoxyribonucleic acid
ET	ethylene
ETI	effector-triggered immunity
GH	glycosyl hydrolase
IG	intersterile group
JA	jasmonic acid
MAPK	mitogen-activated protein kinase
MCO	multicopper oxidase
MEG	malt extracts glucose media
MFS-1	major facilitator superfamily 1
MnP	manganese peroxidase
mRNA	messenger ribonucleic acid
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PRR	pattern recognition receptor
PTI	PAMP-triggered immunity
qPCR	real-time quantitative PCR
R gene	resistance gene
RNA	ribonucleic acid
SA	salicylic acid

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I. Raffaello, T.*, **Chen, H.***, Kohler, A., and Asiegbu, F.O. (2014) Transcriptomic profiles of *Heterobasidion annosum* under abiotic stresses and during saprotrophic growth in bark, sapwood and heartwood. Environmental Microbiology 16: 1654–1667. (*Co-first authors)

II. **Chen, H.**, Kovalchuk, A., Kerio, S., and Asiegbu, F.O. (2013) Distribution and bioinformatic analysis of the cerato-platanin protein family in Dikarya. Mycologia 105: 1479–1488.

III. **Chen, H.**, Quintana, J., Kovalchuk, A., Ubhayasekera, W., and Asiegbu, F.O. (2015) A cerato-platanin-like protein HaCPL2 from *Heterobasidion annosum sensu stricto* induces cell death in *Nicotiana tabacum* and *Pinus sylvestris*. Fungal Genetics and Biology 84: 41–51.

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Author contributions:

I. HC planned the experiment and did the laboratory work together with TR. TR analyzed the data, interpreted the results, and wrote the article. HC also contributed to writing of the article. AK contributed to statistical analysis of the microarray data. FOA conceived the study and contributed to the experimental design and writing of the article.

II. HC designed the study, collected the data, conducted the bioinformatic analysis, and wrote the article. AK contributed to design of the study, bioinformatic analysis, and writing of the article. SK contributed to design of the study, data collection, and writing of the article. FOA conceived the study and contributed to data interpretation and writing of the article.

III. HC designed and planned the experiment, conducted the laboratory work, analyzed the data, interpreted the results, and wrote the article. JQ helped in the laboratory work of recombinant protein expression and bioassay. JQ also contributed to experimental design, data interpretation, and writing of the article. WU did the protein homolog modeling and drafted the related part of the article. AK and FOA conceived the study and contributed to experimental design, data interpretation, and writing of the article.

ABSTRACT

The basidiomycete white-rot fungus *Heterobasidion annosum sensu stricto* (*s.s.*) is one of the most severe conifer pathogens in the Northern Hemisphere. It can live as a saprotroph on dead wood tissues or a necrotroph on living trees. Taking advantage of a sequenced genome from a closely related species, *Heterobasidion irregulare*, we performed an in-depth transcriptomic analysis of this fungus under various abiotic stresses (temperature stress, osmotic stress, oxidative stress, and nutrient starvation) and during saprotrophic growth on pine bark, sapwood, and heartwood. The results unraveled potential regulatory mechanisms to overcome these conditions.

Based on the specific induction in the microarray, cerato-platanins were selected for further study in the interactions between *H. annosum s.s.* and *Pinus sylvestris*. As the first step, a genome-wide bioinformatic study of the cerato-platanin family in Dikarya was therefore conducted. The results suggested that they exist in both Ascomycota and Basidiomycota, but were lost in early branches of jelly fungi as well as in some groups with yeast or yeast-like forms in their life cycles. The ancestor of the Dikarya possessed multiple copies of cerato-platanins, which sorted differently in Ascomycota and Basidiomycota, and this gene family might have expanded in Basidiomycota.

To gain insight into the role of cerato-platanins as potential virulence factors in *H. annosum s.s.*, we performed the functional study of HaCPL2 by using a recombinant protein produced in *Pichia pastoris*. Interestingly, HaCPL2 was able to induce cell death in both host (*P. sylvestris*) and non-host (*Nicotiana tabacum*) plants. Besides cell death symptoms, HaCPL2 retarded apical root growth of *P. sylvestris* seedlings and induced phytoalexin production in *N. tabacum*. Defense-related gene expression was also upregulated in both plants after HaCPL2 treatment.

This study has provided valuable information about potential gene regulatory mechanisms in *H. annosum s.s.* that mediate stress adaptation and growth in different pine wood compartments. Moreover, we propose that HaCPL2, a cerato-platanin protein, could act as an effector and contribute to the virulence in the *H. annosum s.s.–P. sylvestris* pathosystem. This furthers our understanding of *Heterobasidion* pathogenesis and provides a future target for disease control.

1. INTRODUCTION

1.1 Conifer root and butt rot pathogen *Heterobasidion annosum sensu lato* (*s.l.*)

1.1.1 Taxonomy and distribution of the *Heterobasidion annosum sensu lato* (*s.l.*) species complex

The *H. annosum s.l.* species complex is widely distributed in coniferous forests of the Northern Hemisphere, especially in Europe, North America, Russia, China, and Japan (Korhonen and Stenlid, 1998; Korhonen *et al.*, 1998b; Dai *et al.*, 2003; Dai *et al.*, 2006; Otrosina and Garbelotto, 2010). *H. annosum s.l.* species complex comprises five intersterile groups (IGs): *Heterobasidion annosum sensu stricto* (*s.s.*), *Heterobasidion parviporum, Heterobasidion abietinum, Heterobasidion irregulare*, and *Heterobasidion occidentale* (Korhonen, 1978; Capretti *et al.*, 1990; Niemelä and Korhonen, 1998; Otrosina and Garbelotto, 2010). They show different host preferences. In Europe, *H. annosum s.s.* prefers for Scots pine (*Pinus sylvestris*), but also attacks many other conifers and even some broad-leaved trees. *H. parviporum* infects mainly Norway spruce (*Picea abies*), but is also associated with *Abies sibirica, Larix decidua*, and *Pinus cembra*. *H. abietinum* infects mainly silver fir (*Abies alba*). In North America, *H. irregulare* attacks pines, junipers (*Juniperus* spp.), and incense cedar (*Calocedrus decurrens*), while *H. occidentale* has a broad host range (Garbelotto and Gonthier, 2013). In Finland, Scots pines, Norway spruces, and birches are the most economically important trees. Thus, the enormous yearly economic losses to the forest industry due to this pathogen (*H. annosum s.l.*) further merits research on its pathobiology.

1.1.2 Infection biology of *H. annosum s.l.*

The most supported infection model is presented in Figure 1 (Asiegbu *et al*., 2005). Primary infection is mostly mediated by airborne basidiospores deposited on freshly exposed wood surfaces such as stumps, tops, or stem and root wounds. Basidiospores are mainly released during summer when tree stumps are most susceptible to infection (Redfern and Stenlid, 1998). Once the spores geminate, infective hyphae will form and invade the stumps. Later, the fungus may spread and infect surrounding healthy trees by vegetative growth through root contacts or grafts (Garbelotto and Gonthier, 2013). Generally, the nuclear cycle of *H. annosum s.l.* can be described as follows: a basidiospore germinates into a clampless homokaryotic mycelium where all the nuclei are haploid and the cells are multinucleate. A homokaryon is generally weakly virulent and is not able to produce basidiocarps. A basidiospore is incompatible with another spore having the same mating type, but is compatible with all other mating types. Mating with a compatible homokaryon or heterokaryon will result in a heterokaryotic mycelium that is virulent and able to produce fruiting body. The two compatible nuclei fuse in the basidium into a diploid nucleus and subsequent meiosis produces four haploid nuclei, each of which migrates into a basidiospore (Korhonen and Stenlid, 1998).

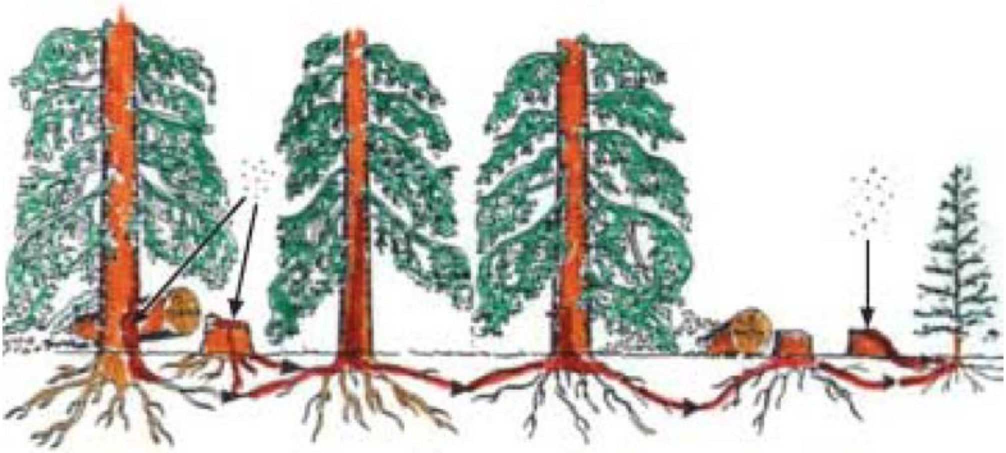

Fig. 1. Infection route of *H. annosum s.l.* in nature. Spores deposit on freshly cut stumps (arrows), germinate, form infective hyphae (red color), and invade the stumps, spreading to neighboring healthy trees by root contact (Asiegbu *et al.*, 2005).

Under *in vitro* laboratory conditions, infection of a Scots pine seedling root normally starts from spore adhesion, germination, and formation of appressorial-like structures within the first two days. From day 3 to 6, necrosis, epidermal penetration, and cortical invasion can be observed. Invasion of the endodermis happens between days 7 and 8, followed by vascular colonization and loss of root turgor, eventually killing the seedling (Asiegbu, 2000). During pathogenesis many cellwall-degrading enzymes (CWDEs) are secreted by *H. annosum s.l.* to facilitate the infection, including amylases, catalases, cellulases, esterases, glucosidases, hemicellulases, manganese peroxidases, laccases, pectinases, phosphatases, and proteases (Huttermann and Cwielong, 1982; Johansson, 1988; Karlsson and Stenlid, 1991; Maijala *et al.*, 1995; Korhonen and Stenlid, 1998; Maijala *et al.*, 2003; Asiegbu *et al.*, 2004). Besides CWDEs, *H. annosum s.l.* also secretes a lot of low-molecular-weight toxins such as fommanoxin, fommanosin, and fommanoxin acid (Basset *et al.*, 1967; Holdenrieder, 1982; Donnelly *et al.*, 1988; Sonnenbichler *et al.*, 1989).

1.1.3 *Heterobasidion* pathogenesis and survival of *H. annosum s.l.* in nature

To successfully colonize a stump, *H. annosum s.l.* must overcome many harsh conditions such as unfavorable temperature and pH, substrate humidity, strong UV radiation, competition from other organisms, and so on. The optimal growth temperature of *H. annosum s.l.* is in the range of 22–28°C (Korhonen and Stenlid, 1998). When the substrate temperature is above 33°C, respiration activity remains low and the fungus loses its ability to infect trees (Asiegbu *et al.*, 2005; Muller *et al.*, 2014). When it is lower than 5°C, the fungus establishes much less frequently, which might be due to the lack of basidiospores, inability to grow at low temperature, or a combination of both

(Meredith, 1959). The optimal growth pH is 4.0–5.7, while conidia germination is unaffected in a wider pH range of 3.0–6.0 (Korhonen and Stenlid, 1998; Avis *et al*., 2009). *H. annosum s.l.* infection rate increases significantly in drier soil relative to wetter soil, where peat forms in cold areas and accumulates organic matter that inhibits the infection (Alexander *et al*., 1975; Korhonen and Stenlid, 1998). The infection rate was much lower in deep peat soils than in mineral soils in long-term experiments carried out in Sitka spruce stands in the United Kingdom (Redfern *et al*., 2010).

In nature, *H. annosum s.l.* has been reported to persist and survive for decades in dead roots left underground after stump removal (Lygis *et al*., 2004b). In these dead tree tissues, the fungus continues to degrade all major components of the wood, including cellulose, hemicellulose, and lignin (Blanchette, 1991; Daniel *et al*., 1998). During *in vivo* growth within woody tissues, as a white-rot fungus, it secretes a wide range of cell wall-degrading enzymes used to exploit a variety of carbon sources (Asiegbu *et al*., 2005). Recently, the genome of *H. irregulare* has been sequenced (Olson *et al*., 2012). The analysis revealed that it encodes a repertoire of lignocellulose-degrading enzymes, including 179 glycoside hydrolases (GHs), 8 manganese peroxidases (MnPs), and 17 multicopper oxidases (MCOs) (Floudas *et al*., 2012; Olson *et al*., 2012). Transcriptomic profiles of *H. irregulare* during necrotrophic and saprotrophic growth have also been compared (Olson *et al*., 2012). Despite the importance of GHs, MnPs, and MCOs in wood degradation, information on genes that are actively and specifically induced during saprotrophic growth on bark, sapwood, and heartwood is still missing in the literature, and very little is known about genes and pathways involved in the specific responses to unfavorable environmental stresses. Thus, this formed the basis for studies conducted in Paper I.

1.1.4 Control strategies for *H. annosum s.l.*

In managed forests, the primary infection of *H. annosum s.l.* usually starts from stump infection followed by vegetative spread through root contacts established between stumps and neighboring trees (Stenlid and Redfern, 1998). Curative measures against the root rot are not feasible because decay inside a tree cannot really be healed. However, disease can be controlled by preventive measures, for example, by restricting or eliminating the infection sources including basidiospore deposition, germination, and growth of the fungus. Current strategies used in practice include silvicultural methods, stump removal, and chemical and biological control (Asiegbu *et al.*, 2005).

Due to differences in resistance against *H. annosum s.l* among conifer species and broad-leaved trees, planting less susceptible species like broad-leaved trees could help to diminish the root problems (Korhonen *et al.*, 1998a; Lygis *et al.*, 2004a; Lygis *et al.*, 2004b). Avoiding planting monocultures of susceptible species is another option. Mixed stands for which less losses have been reported are recommended (Linden and Vollbrecht, 2002). Since basidiospore deposition happens mostly during summer time, winter thinning or cutting can reduce the risk of stumps exposed to the inoculum (Moykkynen *et al.*, 2000; Moykkynen and Miina, 2002). The application of compound fertilizers has been reported to increase resistance of Scots pines against *H. annosum s.l.* (Piri, 2000). Alternatively, the infected stumps can be removed completely. This is an expensive measure, but is considered one of the most effective strategies (Korhonen *et al.*, 1998a). Due to industry supply demands, however, these measures are not always feasible. Chemical control is achieved by spraying urea or borates on the stump surface immediately after tree felling (Pratt *et al.*, 1998). Hydrolysis of urea by ureases from fresh stumps results in production of ammonia that can increase the pH to alkaline, thus inhibiting fungal growth (Johansson *et al.*, 2002). Borates can inhibit the germination of basidiospores and prevent the establishment of the fungus (Pratt and Quill, 1996). Chemical control is cheap, effective, and commercially available. However, negative effects on changes in ground vegetation, soil properties, and fungal community structures have been reported, and the concerns about usage have been raised (Westlund and Nohrstedt, 2000; Vasiliauskas *et al*., 2004). Biocontrol is achieved by application of competitors or antagonists of *H. annosum s.l.* on fresh stumps immediately after tree felling. Currently, *Phlebiopsis gigantea* is used as a biocontrol agent against *H. annosum s.l.*, and commercial products are available (Pratt *et al*., 2000). This method can lead to a satisfactory prevention of the pathogen and seems to be environmentally friendly.

1.2 Plant innate immunity

Plants, unlike mammals that possess both innate and acquired immunity, rely on the innate immunity of each cell and on systemic signals emanating from infection sites, including ISR (induced systemic resistance)/SAR (systemic acquired resistance) and RNAi (Ausubel, 2005). It is widely accepted that plants have developed two layers of innate immunity against pathogen attack, namely PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006). PTI, also termed basal resistance, is activated by the recognition of pathogen-associated molecular patterns (PAMPs) by transmembrane pattern recognition receptors (PRRs) (Jones and Dangl, 2006). In contrast to PTI, ETI is highly strain- or race-specific and is mediated by direct or indirect interaction of pathogen effectors and proteins encoded by resistance genes (R genes), which often leads to programmed cell death and systemic acquired resistance (SAR) in the host (Jones and Dangl, 2006). ETI is effective against pathogens that can grow only on a living host (obligate biotrophs), or hemi-biotrophic pathogens, but not against necrotrophic pathogens that kill host tissue during colonization (Glazebrook, 2005). For necrotrophic pathogens, an alternative model, the so-called inverse gene-for-gene model, has been proposed. The recognition of host-specific toxins (HSTs), a specialized group of necrotrophic effectors, confers susceptibility to the pathogen and leads to HST-induced programmed cell death (Friesen *et al.*, 2008a; Friesen *et al.*, 2008b; Oliver and Solomon, 2010). This model shares similar characteristics with the classic gene-for-gene model, albeit in an opposite manner as described for biotrophs. The classical dichotomy PTI–ETI has been recently reviewed, and a more general and inclusive conceptual model about plant immunity called the "Invasion Model" has been proposed (Cook *et al.*, 2015). The authors consider plant immunity to be a dynamic and continuous system that has evolved to detect invasion. In the Invasion Model, invasion pattern receptors (IPRs) recognize external or endogenous modified ligands, invasion patterns (IPs), to develop IP-triggered responses. These responses do not necessarily result in immunity; they will culminate in continued symbiosis or the end of symbiosis. This new conceptual paradigm expands the principles of the zig-zag model, integrating some limitations of the former, such as explaining necrotroph "pro-death" invasion strategy, as mentioned above.

Early responses to both PTI and ETI include rapid influx of calcium ions, oxidative burst, activation of mitogen-activated protein kinases (MAPKs), changes in protein phosphorylation, phytoalexin biosynthesis, hormone biosynthesis, reinforcement of cell walls at the site of attempted infection, receptor endocytosis, and transcriptional reprogramming (Schwessinger and Zipfel, 2008; Segonzac and Zipfel, 2011). The rapid increase of cytoplasmic Ca^{2+} concentration might serve as a second messenger to promote the opening of other membrane channels or to activate calcium-dependent protein kinases (Blume *et al.*, 2000; Ludwig *et al.*, 2005). The oxidative burst generated in plant–pathogen interactions is associated with hypersensitive response-like cell death, oxidative cross-linking of lignin monolignols that leads to cell wall reinforcement and defense gene expression (Torres *et al.*, 2006; Miller *et al.*, 2009). The activation of MAPK is accompanied by changes in protein phosphorylation, leading to the activation of several families of transcription factors, *e.g.* the WRKY family, which mediates transcriptional activation of defense-related genes (Asai *et al.*, 2002). Phytoalexins are a heterogeneous group of low molecular mass secondary metabolites with antimicrobial activity against a variety of pathogens (Ahuja *et al.*, 2012). The downstream responses to both PTI and ETI are known to be controlled by signaling pathways mediated by hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Bari and Jones, 2009; Robert-Seilaniantz *et al.*, 2011). It has been established that JA and ET-dependent responses are typically involved in the plant defenses against necrotrophic pathogens and insect damage, while SA-dependent defenses are often triggered by biotrophic pathogens (Spoel *et al.*, 2007). In the late responses, pathogen infection not only promotes changes in secondary metabolism to activate defense responses, but also in primary metabolism, with reduced growth and abnormal development in many cases (Berger *et al.*, 2007). Seedling growth inhibition has been reported as a physiological switch from a growth to a defense program in plants (Boller and Felix, 2009).

The production of PR proteins is essential for plant defense. They are usually of small size with a wide range of antimicrobial actions *in vitro* through hydrolytic activities on cell walls, contact toxicity, and perhaps an involvement in defense signaling. Most of them, at least 13 families, are induced by the signaling compounds SA, JA, or ET upon infection with oomycetes, fungi, bacteria, or viruses, as well as during nematode or insect attack (van Loon *et al.*, 2006). To date, 17 families of PR proteins have been recognized and two additional families have been proposed (Custers *et al.*, 2004; van Loon *et al.*, 2006; Sooriyaarachchi *et al.*, 2011). The properties of these families include glycoside hydrolases, chitinases, thaumatins, peroxidases, endoproteinases, putative ribonucleases, proteinase inhibitors, oxalate oxidases, lipid transfer proteins, and small cationic antimicrobial peptides (defensins and thionins) (van Loon *et al.*, 2006). In trees, the induction of 11 PR protein families has been observed upon fungal infection (Veluthakkal and Dasgupta, 2010; Kovalchuk *et al.*, 2013). Although PR proteins play an important role in plant defenses, their production and accumulation are not cost-effective and have a marked impact on plant growth (van Loon *et al.*, 2006).

1.3 Elicitors and effectors from plant pathogens

Plant pathogens have evolved diverse strategies to affect host fitness and create an unbalanced status of symbiosis for their own benefit. To exploit plants as a nutrient source, bacteria usually enter plants through stomata, hydathodes, or wounds and proliferate in the apoplast. Fungi can either penetrate plant epidermal cells directly or grow on top of, between, or through plant cells. Fungi and oomycetes can form feeding structures haustoria with an invagination of host plasma membrane into host cell. All of these pathogens deliver effectors into plant cells to modulate host defense responses and enhance pathogen fitness (Jones and Dangl, 2006). Little is known about the nature of molecules secreted by *H. annosum s.s.* and how they facilitate the infection in their host.

As mentioned above, to successfully infect a plant, pathogens must overcome two layers of plant defense (PTI and ETI). Theoretically, loss of PAMPs that can be recognized by PRRs should favor pathogens evading PTI. However, PAMPs are usually important molecules that evolve slowly and are not easily discarded by the microbes such as bacterial flagellin (flg22), bacterial elongation factor Tu (EF-Tu), glucans, chitins, xylanases, proteins, lipids, and oligosaccharide (Boller and Felix, 2009). Instead of avoiding triggering PTI, successful pathogens deliver effectors into host cells, which contribute to pathogen virulence by affecting host cell structure or function and promoting effector-triggered susceptibility (Jones and Dangl, 2006). These effectors can be apoplastic or cytoplasmic and serve different roles during the invasion process (Wirthmueller *et al.*, 2013). Some may play structural roles, some promote nutrient leakage or pathogen dispersal, and others contribute to suppression of one or more components of PTI or ETI (Schulze-Lefert and Panstruga, 2003). The recognition of PAMPs by PRRs or effectors by products of R genes in plants serves as an invasive signal by potential pathogens. However, plants can perceive another kind of invasive signal, which is called damage-associated molecular patterns (DAMPs). DAMPs are often nuclear or cytosolic proteins with defined intracellular function that are released from the damage or death of host cells (*e.g.* cell wall fragments, cutin monomers, and peptides) (Rubartelli and Lotze, 2007). It is worth mentioning that the pathogenicity and virulence of necrotrophic pathogens, like *H. annosum s.s.*, strongly relies on the secretion of general and host-specific toxins (HSTs). Toxins can be defined as pathogen-derived molecules that can reproduce some or all of the symptoms of infection induced by the intact pathogen when introduced into plants at relevant concentrations (Oliver and Solomon, 2010). HSTs are considered a specialized group of effectors that induce toxicity and promote disease only in host species or in selected genotypes carrying a specific susceptibility allele (Friesen *et al.*, 2008a). If an effector is directly or indirectly recognized by products of R genes, ETI will be triggered and usually result in hypersensitive cell death and disease resistance. However, if a HST is recognized by the host cells, it will result in programmed cell death and lead to disease development because necrotrophic pathogens can feed on dead tissues. Loss or modifying the recognized effectors can help pathogens to avoid ETI, while acquiring additional effectors can help to suppress ETI. Natural selection results in new R specificities so that ETI can be triggered again (Fig. 2).

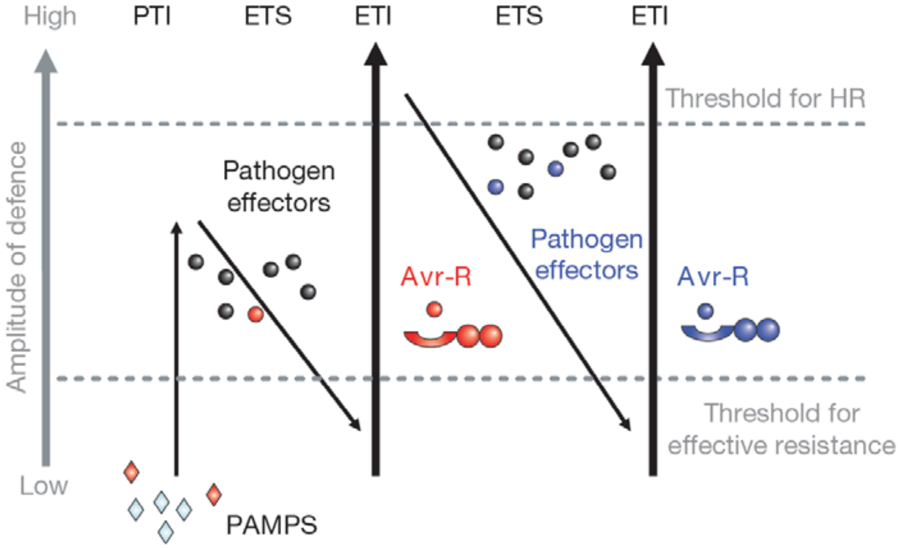

Fig. 2. A zigzag model for the plant immune system (Jones and Dangl, 2006).

So far, despite extensive expression profiling studies (Olson *et al.*, 2012; Raffaello *et al.*, 2014), the contribution of small secreted proteins (SSPs) from *H. annosum s.s.* to pathogenicity and virulence remains poorly understood. Our previous transcriptomic study, where over 12,000 *H. annosum s.s.* genes were analyzed, uncovers one cerato-platanin (*hacpl2*) among the most upregulated transcripts associated with responses to biotic stress (Jaber *et al*., unpublished). However, little is known about their role in the *H. annosum s.s.–P. sylvestris* pathosystem.

1.4 Cerato-platanin: a fungus-specific elicitor or effector?

1.4.1 Discovery of the cerato-platanin family

The first cerato-platanin gene *snodprot1* was identified from the phytopathogenic fungus *Phaeosphaeria nodorum* (anamorph: *Stagonospora nodorum*). The transcripts of *snodprot1* are induced during the infection of wheat leaves (Hall *et al*., 1999). However, the reference protein cerato-platanin has initially been identified in the culture filtrates of the ascomycete *Ceratocystis fimbriata f. sp. platani*, the causal agent of canker stain of European plane trees (Pazzagli *et al*., 1999). The mature protein consists of 120 amino acids and is about 12.4 kDa. It has a high proportion of hydrophobic residues (more than 40%), with four conserved cysteines forming two disulfide bridges, and lacks post-translational modification, except for the removal of a signal peptide and the formation of two disulfide bridges (Pazzagli *et al*. 1999). It is located in the fungal cell wall of ascospores, conidia, and hyphae and is secreted extracellularly (Pazzagli *et al*., 1999; Boddi *et al*., 2004). In addition to this protein, some other family members, such as SnodProt1 of *S. nodorum*, Epl1 of *Trichoderma atroviridis,* Sm1 of *Trichoderma virens,* and BcSpl1 of *Botrytis cinerea,* have also been initially identified as major secreted proteins from fungal culture filtrates (Hall *et al*. 1999, Djonovic *et al*. 2006, Seidl *et al*. 2006, Frias *et al*. 2011).

1.4.2 Multifunctional activities of the cerato-platanin family

Cerato-platanin family proteins have been shown to be multifunctional during fungal growth as well as in fungus–host interactions. *Cerato-platanin* from *C. platani* is involved in hyphal growth and chlamydospore formation. Its expression is downregulated in the condition where fungal growth is reduced and upregulated during chlamydospore formation (Baccelli *et al*., 2012). *Snodprot1* from *Neurospora crassa* is a clock-controlled gene with an expression peak in the morning (Zhu *et al*., 2001). *SP1* from *Leptosphaeria maculans* is regulated by light (Wilson *et al*., 2002). In the interactions between fungi and animals, *CS-Ag* from *Coccidioides immitis* is a pathogen-specific antigen, which is used for infection diagnosis in humans (Pan and Cole 1995). *rAsp f 13* from *Aspergillus fumigatus* is also an allergen in humans (Chaudhary *et al*. 2010). *Snodprot* from *Dactylellina cionopaga* is a parasitism-related protein of nematophagous fungi, which is able to change the chemotaxis and increase the body-bend frequency of *Caenorhabditis elegans* (Yu *et al*. 2012).

Besides the diverse functions mentioned above, research on the cerato-platanin family has primarily focused on the eliciting ability and phytotoxicity of the fungi in interactions with plants. For example, elicitor Sm1 from *T. virens* is able to induce plant defense responses, such as defense-related gene expression and production of reactive oxygen species, and is required for systemic resistance against pathogens (Djonovic *et al*., 2006; Djonovic *et al*., 2007). Other family members, such as CP from *C. platani*, cerato-populin (Pop1) from *Ceratocystis populicola*, MgSM1 from *Magnaporthe oryzae*, and BcSpl1 from *B. cinerea*, are also able to induce plant defense responses, *e.g.* phytoalexin production, and systemic acquired resistance (SAR). Pretreatments of these proteins on plant leaves limit pathogen growth in the whole plant, and in one case it completely inhibited conidia germination (Fontana *et al*., 2008; Comparini *et al*., 2009; Yang *et al*., 2009; Frias *et al*., 2013). The restriction of pathogen growth might be due to the generation of salicylic acid, which expands from the site of protein infiltration, and the overexpression of defense-related genes throughout the plant (Fontana *et al.*, 2008; Frias *et al.*, 2013). In contrast to their role in inducing resistance against plant pathogens, some proteins of this family, especially from pathogenic fungi, have been demonstrated to be phytotoxic and to contribute to full virulence of the pathogens. For instance, CP from *C. platani*, BcSpl1 from *B. cinerea,* and MpCP1 from *Moniliophthora perniciosa* are induced during infection and are able to cause hypersensitive cell death (HCD) or necrosis on plants (Pazzagli *et al.*, 1999; Frias *et al.*, 2011; de O. Barsottini *et al.*, 2013). Knock-out mutants of *bcspl1* from *B. cinerea* show reduced virulence in a variety of hosts (Frias *et al.*, 2011).

1.4.3 Protein structures of the cerato-platanin family

The eliciting ability, phytotoxicity, and other biological functions of the cerato-platanin family might be attributed to their protein structures, self-aggregation ability, and existing forms. Three-dimentional structures of several cerato-platanins (CP from *C. platani*, Sm1 from *T. virens*, MpCP1, MpCP2, MpCP3, and MpCP5 from *M. perniciosa*) show a double-ψβ-barrel fold remarkably similar to those found in plant and bacterial expansins, lytic transglycosylases (LTs), endoglucanases, formate dehydrogenase H, dimethyl-sulfoxide reductases, aspartic proteinases, and plant defense protein barwins (de Oliveira *et al.*, 2011; de O. Barsottini *et al.*, 2013; Pazzagli *et al.*, 2014). Interestingly, similar to expansins, cerato-platanin is able to bind *N*-acetylglucosamine oligomers, chitin, and colloidal chitin and loosen cellulosic materials via a non-enzymatic mechanism (de Oliveira *et al.*, 2011; Baccelli *et al.*, 2014a). Residues involved in oligosaccharide-binding activity are well conserved in the family, suggesting that it might play a role in polysaccharide recognition (de Oliveira *et al.*, 2011; Frischmann *et al.*, 2013). Moreover, proteins from this family exist as different forms, *e.g.* monomers, dimers, trimers, polymers, or a mixture of these, when secreted into media (Seidl *et al.*, 2006; Vargas *et al.*, 2008; Zaparoli *et al.*, 2009). In *T. virens*, the eliciting ability of Sm1 is controlled by dimerization, with only the monomeric form able to induce systemic resistance in plants, and glycosylation is the key factor preventing dimerization of Sm1 (Vargas *et al*., 2008). However, this does not agree with Martellini's observation that the aggregate form shows higher eliciting ability in inducing plant defense responses (Martellini *et al*., 2013). Furthermore, the phytotoxicity of BcSpl1 is found to reside in a two-peptide motif on the protein surface (Frias *et al*., 2014). The two disulfide bridges formed by the four conserved cysteines are important for protein conformation. Reduction and derivatization of the –SH groups causes a loss of solubility and eliciting ability (Pazzagli *et al*., 2006).

Cerato-platanins, like other fungal surface proteins, can self-aggregate *in vitro* and form annular-shaped oligomers, which eventually turn into amyloid-like aggregates through a nucleated-growth mechanism (Carresi *et al*., 2006; Sbrana *et al*., 2007; Vargas *et al*., 2008; Comparini *et al*., 2009; Pazzagli *et al*., 2009). These aggregates can interact with hydrophobic surfaces, such as the cuticle of leaves, and induce plant hypersensitive responses (Pazzagli *et al*., 2009; Frischmann *et al*., 2013; Martellini *et al*., 2013). Their self-assembly at air/water interfaces can increase the polarity of aqueous solutions and surfaces, in contrast to hydrophobins (Frischmann *et al*., 2013). The ability to form aggregates varies among members of this family, showing a negative correlation with the presence of glycosylation sites (Vargas *et al*., 2008).

The expansin-like activity, eliciting ability, and phytotoxicity of cerato-platanins are summarized in Figure 3 (Pazzagli *et al*., 2014).

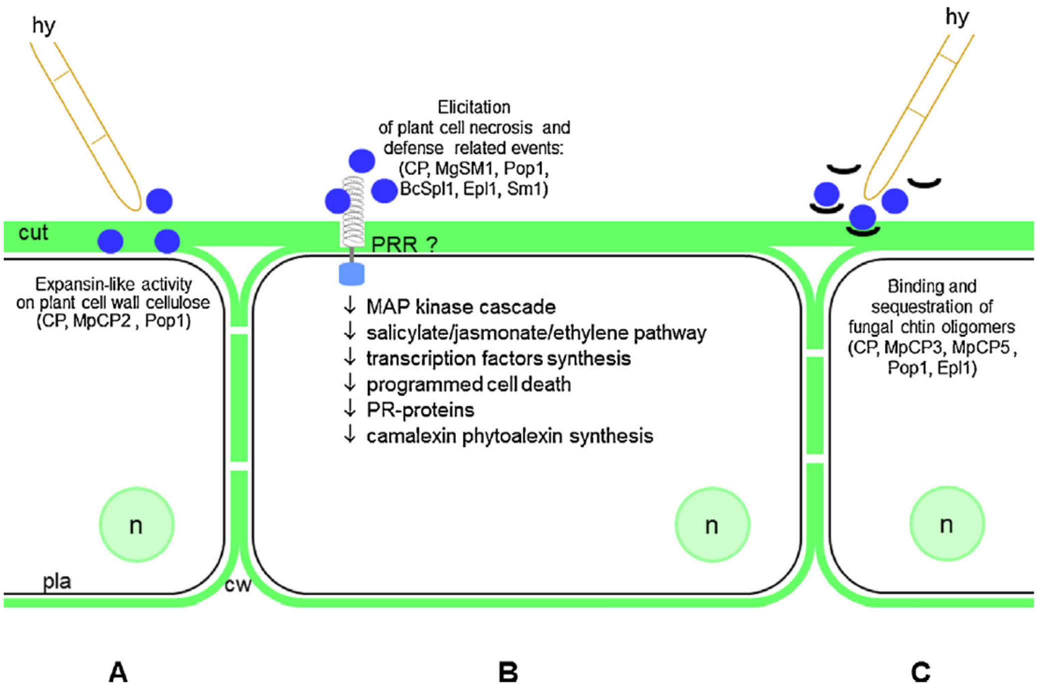

Fig. 3. Schematic representation of possible interaction scenarios between cerato-platanin proteins and plants (blue circle: CP-like proteins; black moon shape: fungal oligo-*N*-acetylglucosamine produced by hypha after plant interaction; ?: hypothetical; hy: fungal hyphae; cut: plant cuticle; cw: plant cell wall; pla: plant cell plasma membrane; n: plant cell nucleus; PRR: plant pattern-recognition receptor) (Pazzagli *et al*., 2014).

2. AIMS OF THE STUDY

H. annosum s.s. is a major causal agent of root and butt rot disease of conifers in the Northern Hemisphere. As one of the most economically important forest pathogens, *H. annosum s.s.* presents a well-characterized but complex lifestyle. It can live as a saprotroph or a necrotroph and switch its lifestyle depending on substrate availability. Many studies have been conducted to understand its biology, ecology, impact, and control strategy. However, little is known about the molecular mechanisms controlling its survival in a harsh environment and its pathogenesis. In this study, with the aid of a sequenced genome of a closely related species, *H. irregulare*, we investigated the regulatory mechanisms of *H. annosum s.s.* under various conditions, with specific aims as follows:

- To evaluate the transcriptomic profiles of *H. annosum s.s.* under various abiotic stresses (temperature stress, osmotic stress, oxidative stress, and nutrient starvation) and during saprotrophic growth on different pine wood materials (bark, sapwood, and heartwood) (Paper I).
- To perform a genome-wide bioinformatic analysis to investigate the distribution of the cerato-platanin family in Dikarya and the correlation of homolog number and fungal nutrition modes. This is a protein family with special interests, members of which behave as fungal elicitors or effectors (Paper II).
- To investigate the function of cerato-platanins during interactions between *H. annosum s.s.* and *P. sylvestris* (Paper III).

3. MATERIALS AND METHODS

Materials and methods used in this study are summarized in Table 1 and Table 2. Detailed descriptions can be found in the original publications.

Table 1. Strains and plant materials used in this study.

Strains and plant materials	Publications
Heterobasidion annosum sensu stricto (isolate 03012, heterokaryon)	I, III
TOP10F´ *Escherichia coli*	III
Pichia pastoris X-33 (wild-type, Mut$^+$)	III
Nicotiana tabacum cv.SR1	III
Pinus sylvestris L., Saleby FP-45	III

Table 2. Methods used in this study.

Methods	Publications
Growth conditions of *H. annosum s.s.*	I, III
RNA isolation	I, III
RNA purification and amplification	I
cDNA preparation for microarray	I
Microarray preparation and data analysis	I
Primer design	I, III
Quantitative real-time PCR preparation and data analysis	I, III
Bioinformatic analysis of cerato-platanin protein family	II

Genomic DNA isolation	III
Gene cloning	III
Recombinant protein expression in *P. pastoris*	III
Protein purification and identification	III
Bioassay of recombinant protein on *N. tabacum* and *P. sylvestris*	III
Homology modeling	III

4. RESULTS AND DISCUSSION

4.1 Transcriptomic profiles of *H. annosum sensu stricto* (*s.s.*) under abiotic stresses and during saprotrophic growth on pine bark, sapwood, and heartwood (I)

The ability of fungi to sense and survive in a diverse range of environmental stresses and to actively engage in nutrient acquisition has been a central issue in fungal biology research. In nature, primary infection of *H. annosum s.s.* is mostly mediated by airborne basidiospores on freshly exposed wood surfaces, such as stump surfaces or stem and root wounds, which are exposed to harsh environmental conditions (Garbelotto and Gonthier, 2013). To successfully establish and colonize on these surfaces, the fungus must overcome several critical abiotic stresses. Moreover, when basidiospores land on wood surfaces, due to the different properties of pine bark, sapwood, and heartwood, the fungus might need to exploit different mechanisms in order to colonize on wood materials.

With the advantage of a sequenced genome of the closely related species *H. irregulare*, a transcriptomic study of *H. annosum s.s.* under abiotic stress (temperature stress (8°C and 27°C for 3 weeks), osmotic stress (0.5 M of either NaCl or $CaCl_2$ for 60 min), oxidative stress (5 mM H_2O_2 for 60 min), and nutrient starvation (growth in 0.2 g/ml glucose solution for 5 days)) and during saprotrophic growth in different pine wood materials (bark, sapwood, and heartwood for 3 months) was conducted using the growth in MEG media as a control. Finally, an independent qPCR analysis of 51 genes was carried out to further validate the expression in the microarray study.

4.1.1 General overview of transcriptomic data (I)

The hierarchical cluster analysis of the transcriptomic profiles showed that all of the biological replicates clustered together, indicating reproducibility and a consistent transcriptional pattern within the biological replicates (I, Supporting Information Fig. 1). It also revealed a first level of separation between saprotrophic growth on pine wood and abiotic conditions. There were also separate clades for control and temperature stress, osmotic stress, and oxidative stress and nutrient starvation (I, Supporting Information Fig. 1). The presence of evident separation in the cluster analysis indicates different fungal responses and adaptation to these diverse conditions.

The comparative transcriptome analysis revealed that compared with the control 13 genes were upregulated at both 27°C and 8°C, 39 in both $CaCl_2$ and NaCl, and 57 in both H_2O_2 and nutrient starvation. In pine wood, 529 genes were induced in all wood compartments. Bark and heartwood material induced similar numbers of specific transcripts in *H. annosum s.s.* (148 and 174, respectively). However, a total of 448 transcripts were specifically induced during fungal growth on sapwood (I, Fig. 1). Probably the high content of easily utilizable sugars and polysaccharides in the sapwood facilitates the induction of an optimal set of genes to allow the fungus to utilize these carbon sources effectively (Terziev *et al.*, 1997). This may indicate an evolutionary adaptation of *H. annosum s.s.* for pine sapwood degradation compared with heartwood and bark, which contain more fungistatic compounds (Asiegbu *et al.*, 2005).

4.1.2 Response of *H. annosum s.s.* to temperature stress (I)

The general physiological adaptation of fungi to low temperature is characterized by accumulation of trehalose and cryoprotectant sugars, *e.g.* glycerol and mannitol, changes in the lipid membrane composition with an increase of unsaturated fatty acids, and production of anti-freeze proteins (Robinson, 2001). In this study, several genes involved in lipid metabolism were affected during temperature stress.

Compared with the control, a *phospholipid methyltransferase* similar to the yeast *PEM2* involved in the synthesis of phosphatidylcholine from phosphatidylethanolamine was strongly induced at 8°C, indicating that enrichment of the fungal membrane in phosphatidylcholine may be important in cold stress adaptation (I, Fig. 2 A) (Kodaki and Yamashita, 1987). Several *cytochrome P450s* and up to 17 genes of the *major facilitator superfamily 1* (*MFS-1*) were also found to be upregulated in temperature stress (I, Fig. 2 A). The cytochrome P450 is a superfamily of monooxygenases capable of oxidizing a variety of endogenous or exogenous substrates (Meunier *et al*., 2004), and MFS-1 family proteins are predicted to be transmembrane transporters (antiporters, symporters, and uniporters) of a wide range of substrates (Marger and Saier, 1993; Law *et al*., 2008). The induction of both *cytochrome P450* and *MFS-1* genes indicates that these monooxygenases may take part in intracellular pathways required for temperature adaptation or are involved in detoxification.

4.1.3 Response of *H. annosum s.s.* to osmotic stress (I)

The *H. annosum s.s.* response to osmotic stress was investigated by exposure of the fungal mycelia to a high concentration of NaCl and $CaCl_2$. $CaCl_2$ exposure was characterized by a significantly higher number of upregulated genes (415) than in NaCl exposure (89) (I, Fig. 1B). Twenty *protein kinases* were induced, especially in $CaCl_2$ (I, Fig. 2 B). We have previously demonstrated the activation of the *H. annosum s.s*. protein kinase HaHog1p under NaCl stress conditions (Raffaello *et al*., 2012). A predicted gene similar to the *Saccharomyces cerevisiae glycerol-3-phosphatase* (*GPP2)* was also induced (I, Fig. 2 B). *GPP2* is triggered by Hog1p phosphorylation, and glycerol is accumulated as an osmolyte in *S. cerevisiae* under osmotic stress (Hohmann, 2002). This suggests *H. annosum s.s.* may also use glycerol as an osmoprotectant. Type-3 copper tyrosinases are key components in melanin biosynthesis, and melanization of the fungal cell wall has been associated with stress response to harsh environmental conditions (Bell and Wheeler, 1986; Halaouli *et al*., 2006). A strong induction of a *tyrosinase* may indicate activation of the melanin biosynthesis pathway and reinforcement of the cell wall by melanization in *H. annosum s.s.* under osmotic stress (I, Fig. 2 B). This could be important in responding to the high level of Ca^{2+} that accumulates in decayed wood and reaction zones in infected trees (Oliva *et al*., 2011; Nagy *et al*., 2012). The induction of a predicted *vacuolar* Ca^{2+}/H^{+} *antiporter* also suggests a general fungal response to keep the intracellular Ca^{2+} concentration at a low level by sequestration into the vacuolar compartment (I, Fig. 2 B).

4.1.4 Response of *H. annosum s.s.* to oxidative stress and nutrient starvation (I)

Under oxidative stress, *cytochrome P450* and *flavin oxidoreductase* were induced (I, Fig. 2 C), which are involved in intracellular redox activity (Huijbers *et al*., 2014). This could indicate a general response to the high level of oxidants. The induction of *UvrD/REP helicase* and *Rad1* (I, Supporting Information Table 11) indicates the activation of pathways involved in DNA repair caused by a high level of oxidants (Chene, 2008).

Several genes involved in glyoxylate metabolism (*malate synthase*, *citrate synthase*, and *isocitrate lyase*) and two *phosphatidylinositol-4-phosphate 5-kinases* (*PIP5K*) were specifically induced during starvation (I, Fig 2 C). In eukaryotic cells, activation of autophagy is a way for the cell to respond to nutrient deprivation. In yeast, the formation of autophagosomes is activated by phosphatydilinositol-3-kinase (PtdIns3K), which are used to degrade cellular components in order to recycle macromolecules (He and Klionsky, 2009). *Candida albicans* has been shown to overexpress genes involved in the glyoxylate cycle, like *isocitrate lyase* and *malate synthase*, to survive in starving conditions inside the phagosomes of macrophage cells (Lorenz and Fink, 2001).

4.1.5 Response of *H. annosum s.s.* during saprotrophic growth on pine bark, sapwood, and heartwood (I)

The growth of *H. annosum s.s.* on pine wood compartments stimulated the production of different enzymes for wood degradation. The carbohydrate-active enzymes (CAZy) specifically induced in different wood materials are listed in Supporting Information Table 12 (I). The *glycosyl hydrolases* (*GHs*) are among the largest gene families induced during wood colonization. *GH* genes encode diverse enzymes that hydrolyze the glycosidic bonds that characterize cellulose, hemicellulose, and pectin. A total of 31 predicted *GH* genes were found to be upregulated in heartwood, 20 in sapwood, and 23 in bark compared with the control (I, Fig. 3). Some genes of these *GH* families (*GH1*, *GH3*, *GH15*, *GH18*, *GH28*, *GH30*, *GH35*, *GH53*, and *GH88*) showed general induction in all wood compartments, while some members of other *GH* families (*GH5*, *GH10*, GH12, *GH45*, and *GH61*) displayed strong induction only in heartwood (I, Supporting Information Fig. 2). Enzymes from GH61 do not possess the typical glycosyl hydrolase activity. However, they support the activity of the other GH enzymes through an oxidative degradation of cellulose (Harris *et al*., 2010; Phillips *et al*., 2011; Quinlan *et al*., 2011; Levasseur *et al*., 2013). Interestingly, no *GH* was specifically induced only in bark or only in sapwood. The results highlight the fine regulation of distinct gene families in *H. annosum s.s.* that are pivotal for wood degradation, especially for heartwood degradation.

Besides *GH* genes, 5 *manganese peroxidases* (*MnPs*), 1 *cytosolic oxidoreductase*, and 3 *multi-copper oxidases* (*MCOs*) were found to be specifically induced during saprotrophic growth (I, Fig. 5). These enzymes together with lignin peroxidases (LiPs) are the main enzymes involved in lignin degradation in white-rot fungi (Aro *et al*., 2005). Besides the hydrolysis of cellulose and its associates, lignin degradation is another essential part of wood degradation. Lignin is a heterogeneous aromatic polymer that protects cellulose and hemicellulose from microbial attack (Floudas *et al*., 2012). Thus, to gain access to cellulose or hemicellulose, wood-decaying fungi must overcome or circumvent lignin (Floudas *et al*., 2012). In an earlier study, only one *MnP* and one *MCO* is upregulated during wood degradation in *H. irregulare* (Olson *et al.*, 2012). The differences could be attributed to variations in fungal species and experimental design; in their study, *H. irregulare* was grown on whole pine wood shavings. Unlike the earlier study, the current results provide additional details on the gene expression pattern when the fungus navigates specific wood compartments during the saprotrophic wood decay process. The expression of *MCOs* and *MnPs* has been reported to be regulated by environmental signals such as concentration of carbon and nitrogen, metal ions, presence of xenobiotics, temperature shock, and length of daylight (Janusz *et al.*, 2013). Thus, the chemical composition of different wood compartments might lead to selectively induced expression of ligninolytic enzymes. Furthermore, the co-expression of the ligninolytic enzymes are also reported in *Phlebia radiata* when wood was used as substrate (Hilden *et al.*, 2006; Makela *et al.*, 2006; Lundell *et al.*, 2010). These results have provided insight into the specific roles and importance of paralogous genes encoding MCOs and MnPs in pine wood degradation.

4.1.6 Conclusions for Study I

In this study, the transcriptional response of the basidiomycete *H. annosum s.s.* to different abiotic stresses and during saprotrophic growth on specific pine wood compartments was investigated. The balance between sensing and survival in abiotic stresses and nutrient uptake during saprotrophic growth for *H. annosum s.s.* is summarized in Figure 6 (I). Possibly, *H. annosum s.s.* might change the lipid and protein composition in the plasma membrane to adapt to cold stress. The synthesis and accumulation of glycerol as osmoprotectant controlled by MAPK Hog1p, the melanization of fungal cell wall, and the use of vacuolar Ca^{2+}/H^{+} antiporter could be relevant for survival under osmotic stress. Additionally, the activation of the MAPK HOG pathway and DNA repairing pathway might be important for adaptation to oxidative stress. Furthermore, the activation of the glyoxylate cycle and autophagy might be essential for the fungus to overcome nutrient starvation, and the induction of a specific set of enzymes (GHs, MCOs, MnPs, cytosolic oxidoreductases) could be highly relevant for pine wood degradation.

4.2 Distribution and bioinformatic analysis of the cerato-platanin family in Dikarya (II)

The cerato-platanin family is a group of small secreted cysteine-rich proteins exclusive for filamentous fungi. Recent studies have revealed that these proteins are involved in the interactions between fungi and their hosts, acting as elicitors or effectors. However, little is known about the distribution and evolution of this protein family in fungi. The distribution will provide valuable information about whether they are correlated to a certain group of fungi, for instance, according to fungal taxonomy, nutritional types, or living forms. This will help to predict their function. Furthermore, the homolog numbers in each species together with the phylogenetic analysis will shed light on the evolution of this protein family in fungi. We performed a genome-wide distribution and evolution study of this family in fungi and analyzed the common characteristics of their protein sequences. A total of 55 fungal genomes, including 27 from Ascomycota and 28 from Basidiomycota, were used. Altogether 130 cerato-platanin homolog protein sequences were obtained and analyzed.

4.2.1 Distribution of cerato-platanin family proteins (II)

The cerato-platanin family appears to be specific to fungi in that we have not found any homologs in non-fungal species. In Ascomycota, cerato-platanin homologs exist in all classes studied, except the subphyla Taphrinomycotina and Saccharomycotina. They appear as a single-copy gene in most of the species. Furthermore, the distribution in Ascomycota seems to be irrelevant to the nutritional mode because the homologs exist in five different nutritional types (II, FIG. 1). In

Basidiomycota, cerato-platanin homologs exists only in Agaricomycetes and are absent from all other subphyla or classes (Pucciniomycotina, Ustilaginomycotina, Tremellomycetes, and Dacrymycetes). In addition, homolog number varies widely from 1 to 12 (II, FIG. 1). Taphrinomycotina is a subphylum that includes yeast species, dimorphic taxa, and a filamentous sporocarp-producing genus, *Neolecta* (Liu *et al*., 2009). Saccharomycotina, also referred to as true yeasts, contains most of the unicellular ascomycete yeasts (James *et al*., 2006). Fungi in Pucciniomycotina, Ustilaginomycotina, and Tremellomycetes have dimorphic life cycles that include a unicellular haploid yeast phase and a dikaryotic filamentous phase (Oberwinkler, 1987; Millanes *et al*., 2011). Tremellomycetes, Dacrymycetes, and Agaricomycetes contain three groups of jelly fungi and mushrooms in Agaricomycotina (Hibbett, 2007). Our results suggest that during evolution, cerato-platanins appear to have been lost in the early branches in the tree of life for Dikarya (Floudas *et al*., 2012). In Basidiomycota, cerato-platanins exist only in Agaricomycetes, but probably were lost in the early branches of jelly fungi in Tremellomycetes and Dacrymycetes and species that have yeast or yeast-like forms in their life cycle. This observation leads to speculation that cerato-platanins might be involved in sporocarp formation because Agaricomycetes produce diverse forms of basidiocarps. Furthermore, the homolog number seems to have no connection with fungal nutritional type, indicating that cerato-platanin is not specific for certain feeding strategies.

4.2.2 Phylogenetic analysis of the cerato-platanin family (II)

The phylogenetic analysis revealed that multiple independent lineages of ascomycete sequences nested within the basidiomycetes, together with the existence of a homolog in *Gonapodya prolifera*, a very early diverging species of fungi, suggesting that there were multiple copies of cerato-platanins in the ancestor of the Dikarya that have sorted differently in these two groups (II, FIG. 2). Moreover, no specific cluster was found for cerato-platanins from pathogenic fungi, indicating that the involvement in pathogenesis by members of this family might evolve from general functions (II, FIG. 2). There was no expansion of this gene family in Ascomycota, while recent gene duplications were observed in many species in Basidiomycota, which represent all orders in Agaricomycetes (II, FIG. 2). In one study, cerato-platanins were found to be under positive selection in *Botrytis* (Aguileta *et al*., 2012). Whether the higher sequence diversity is correlated with functional diversity is not clear because until now the functional study of the cerato-platanin family has focused on its eliciting ability and phytotoxicity. Only limited studies have investigated other functions for this family such as involvement in hyphal growth and chlamydospore formation (Baccelli *et al*., 2012). This together with the fact that cerato-platanins also exist in non-pathogenic fungi indicate that the protein functions are not limited to pathogenesis.

4.2.3 Sequence characterization of cerato-platanin family proteins (II)

The cerato-platanin family is a group of small secreted cysteine-rich proteins. Our protein sequence comparison has confirmed this description. Secretion signal is observed in 95% of the sequences. The length of the mature proteins is mostly from 105 to 134 amino acids (II, TABLE I). Four cysteines are found in 90% of the sequences (II, TABLE II), which are well conserved and reported to be involved in the formation of two disulfide bridges (Pazzagli *et al*., 1999). A signature sequence CSD/CSN is also found, which is well aligned with the consensus motif CCN of hydrophobins (Wosten, 2001; Pazzagli *et al*., 2006). This family usually contains a high proportion of hydrophobic amino acids, with an average of 37%. They are well conserved and believed to contribute to the self-assembly ability at air/water interfaces, where cerato-platanins form polymeric and amphipathic monolayers (Carresi *et al*., 2006; Frischmann *et al*., 2013). Besides the hydrophobic amino acids, 11 glycines are highly conserved in all homologs (II, TABLE III). Glycines often are found at the turns of a protein backbone and hydrophobic amino acids on the inner side of a protein structure. This suggests that proteins in the cerato-platanin family might share a similar topology. Furthermore, all homolog sequences contain the cerato-platanin domain (IPR010829), 90% contain a barwin-related endoglucanase domain (IPR009009), and 15% contain a barwin-like endoglucanase domain (IPR014733). The 3D structure of cerato-platanin has recently been elucidated. It contains a double-ψ-barrel fold similar to those found in expansins, endoglucanases, and barwin (de Oliveira *et al*., 2011).

4.2.4 Conclusions for Study II

Protein sequence analysis has confirmed that the cerato-platanin family is indeed a group of small secreted cysteine-rich proteins. The distribution of the family proteins further reveals that they are exclusive to filamentous fungi. The phylogenetic analysis suggests that the ancestor of the Dikarya possessed multiple copies of cerato-platanins, which sorted differently in Ascomycota and Basidiomycota. Moreover, the gene family is lost in early branches of jelly fungi as well as in some groups with yeast or yeast-like forms in their life cycle, while it might have expanded in Basidiomycota, which leads to a higher sequence diversity in Basidiomycota than Ascomycota. However, no correlation was found between the homolog number of this family and fungal nutritional types.

4.3 A cerato-platanin-like protein HaCPL2 from *H. annosum s.s.* induces cell death in *Nicotiana tabacum* and *Pinus sylvestris* (III)

H. annosum s.s. can live as a saprotroph or a necrotroph, switching its lifestyle depending on substrate availability (Garbelotto and Gonthier, 2013). As a necrotrophic fungus, it secretes cell wall-degrading enzymes and toxins (fommanoxin, fommanosin, fommanoxin acid, oosponol, and oospoglycol), which can modify host cell functions. Many of these molecules might contribute to the virulence of the pathogen (Asiegbu *et al*., 2005). Although several efforts have been made towards a deeper understanding of the pathogenesis mechanism, no single effector or necrosis-inducing molecule has been functionally characterized in this conifer pathogen. In this study, a cerato-platanin family protein HaCPL2 was investigated, selected based on the results of our previous transcriptomic study by microarray. The analysis included the expression profiles of cerato-platanins by qPCR, over-expression and purification of the recombinant protein HaCPL2, and the eliciting and necrosis-inducing ability in host and non-host plants.

4.3.1 HaCPL2 showed the highest sequence similarity to cerato-platanin from *C. platani* and was induced during nutrient starvation and necrotrophic growth (III)

The reference protein cerato-platanin from *C. platani* contains 134 amino acids with a signal peptide of 14 amino acids (Pazzagli *et al.*, 2006). The three cerato-platanin genes from *H. annosum s.s.*, *hacpl1*, *hacpl2* and *hacpl3*, are 318 bp, 417 bp, and 783 bp, encoding proteins of 105, 138, and 260 amino acids, respectively. However, only *hacpl2* and *hacpl3* contain a putative secretion signal, and HaCPL3 had more than 100 extra amino acids in the *N*-terminal region with low complexity. Based on the protein sequence comparison, HaCPL2 showed the highest similarity to the reference protein (III, Supplementary Fig. 1). Moreover, expression profile by qPCR showed that all of them were induced during nutrient starvation compared with the control, where only a limited amount of carbon was supplied and nitrogen was completely removed (III, Fig. 1A). This indicates that HaCPLs from *H. annosum s.s.* might share a common function that is involved in overcoming the starvation condition. Furthermore, *hacpl2* was significantly induced during necrotrophic growth (III, Fig. 1B), reflecting that HaCPL2 might be involved in the interactions between the fungus and living plants.

4.3.2 Recombinant HaCPL2 existed as a mixture of different forms (III)

Recombinant overexpression of HaCPL2 was carried out in *P. pastoris* due to the low efficiency in constructing a stable gene-specific mutant in *H. annosum s.s.* (Samils *et al.*, 2006). The mature recombinant protein was expected to be 142 amino acids long, including a c-myc and a 6×His tag at the *N*-terminal, and the predicted molecular weight was 14943.48 Daltons. The best expression was obtained when the culture was started with cells at culture optical densities (OD) of 3.5 and induced with 2% methanol for five days. SDS-PAGE electrophoresis revealed that the purified recombinant HaCPL2 existed as a mixture of different forms (monomer, dimer, and polymer) (III, Fig. 2B), and this was confirmed by Western blot (III, Fig. 2C) and mass spectrometry analysis MALDI-TOF-TOF (III, Supplementary Fig. 2). This is similar to the observations for other cerato-platanins (monomer and aggregate forms in *C. platani*, monomer and dimer in *T. virens* and *Trichoderma atroviride*, monomer and trimer in *Trichoderma reesei*, and monomer, dimer, and trimer in *M. oryzae*) (Vargas *et al.*, 2008). In *T. virens*, the eliciting ability of Sm1 has been found to be controlled by dimerization, with only the monomeric form able to induce systemic resistance in plants, and glycosylation is the key factor that prevents dimerization (Vargas *et al.*, 2008). Typically, the yield of pure recombinant HaCPL2 was about 16 mg from 1 L of culture supernatant. This is comparable to other studies using *P. pastoris* to express cerato-platanins (Carresi *et al*., 2006; Buensanteai *et al*., 2010; Frias *et al*., 2011).

4.3.3 HaCPL2 induced cell death in *N. tabacum* leaves and Scots pine seedling roots (III)

The effect of purified HaCPL2 was tested on the model plant *N. tabacum*, a well-established system for elicitor and effector studies, and a native host, Scots pine. Interestingly, HaCPL2 was able to induce cell death in both *N. tabacum* leaves and Scots pine seedling roots, and the symptoms in the latter were similar to those caused by *H. annosum s.s.*. The symptoms were induced at a concentration of 120 µM or more, but not at 38 µM, suggesting that the phytotoxicity was related to concentration (III, Fig. 3A, Fig. 5A and B). Moreover, HaCPL2 also induced phytoalexin production in *N. tabacum* leaf disks and retarded the root growth of Scots pine seedlings at a concentration of 120 µM or more (III, Fig. 3B and C, Fig. 5C). Phytoalexin production was induced in a dose-dependent manner, which correlated well with the severity of the symptoms displayed on the leaf disks (III, Fig. 3B and C). As a response to pest and pathogen attack, plants are able to produce low molecular mass secondary metabolites with antimicrobial activity, which are collectively known as phytoalexins. Therefore, phytoalexin production is an indication of early plant defenses (Ahuja *et al*., 2012). The significantly reduced apical root growth might be related to cell death in the meristem and a physiological switch from a growth to a defense program in the plant (Boller and Felix, 2009). However, no significant differences were observed for the growth of the upper part of the seedlings, indicating that either inhibition was limited at the roots or the incubation time was insufficient to observe disease symptoms on the foliage (III, Fig. 5A). This suggests that HaCPL2 contributes to the virulence of *H. annosum s.s.*.

The eliciting ability seems to be a common characteristic of this protein family. They are able to induce immune responses in both animals and plants. CS-AG from *C. immitis* is an antigen and rAsp f 13 of *A. fumigatus* is an allergen to humans (Pan and Cole, 1995; Chaudhary *et al*., 2010). Cerato-platanins from the genus *Trichoderma*, such as Epl1 from *T. atroviride* and Sm1 from *T. virens*, have been found to act as elicitors, without necrosis-inducing ability (Djonovic *et al*., 2006; Seidl *et al*., 2006; Djonovic *et al*., 2007). Cerato-platanins from some pathogens, such as CP from *C. platani*, MSP1 from *M. oryzae*, Pop1 from *C. populicola*, MpCP1 from *M. perniciosa*, and BcSpl1 from *B. cinerea*, are able to induce necrosis, programmed cell death, or different plant defense responses (Pazzagli *et al*., 1999; Scala *et al*., 2004; Jeong *et al*., 2007; Comparini *et al*., 2009; Zaparoli *et al*., 2009; Frias *et al*., 2011). Recent research has revealed that cerato-platanins are able to bind chitin and loosen cellulosic materials non-hydrolytically as expansins (Baccelli *et al*., 2014a). This might be one of the explanations for their active role in disease development. Their classification as elicitors or effectors following the classical PTI–ETI dichotomy is controversial since the presence of two well-conserved motifs across genera resembles MAMP-like characteristics, albeit those motifs contribute to pathogens' virulence (Frias *et al*., 2014; Cook *et al*., 2015). In this study, we demonstrated that cerato-platanin-like protein HaCPL2 from *H. annosum s.s.* was able to cause cell death and defense responses in host and non-host plants, reproducing some or all of the symptoms of infection induced by the intact pathogen when introduced into the host plant. Since *H. annosum s.s.* is a necrotrophic pathogen, it normally kills living cells before colonization and feeds on dead tissues (Asiegbu *et al*., 2005). Therefore, HaCPL2 might be one of the molecules that *H. annosum s.s.* exploits to induce cell death in plants, thus achieving successful colonization.

4.3.4. HaCPL2 induced defense gene expression in *N. tabacum* leaves and Scots pine seedlings (III)

In *N. tabacum*, the RT-qPCR results showed that in the early response (6 h after infiltration), *PR1a* (a marker of SA-mediated signaling pathway), *PAL* (phenylpropanoid pathway), *PR10* (a marker of hypersensitive cell death), and *PAD3* (camalexin biosynthesis pathway) were significantly upregulated. *PR2a* (a marker of SA-mediated signaling pathway) and *LOX* (JA-mediated signaling pathway) were induced only at a later time point of 24 h. However, a marker gene of the ET-mediated signaling pathway *ERF1* was not induced in leaves treated with HaCPL2 (III, Fig. 4A). Another gene related to the JA-mediated signaling pathway *COI1* was significantly downregulated in a time-dependent manner (III, Fig. 4B).

In Scots pine, expression of several marker genes related to the JA-mediated signaling pathway (*JAZ1*, *OPR* and *ERF1*), ET (*ACS* and *ERF1*) and terpene biosynthesis (*TPS*), cell wall modification (*PME* and *LAC*), and SA-mediated signaling pathway (*PAL1*, *NPR1* and *LURP1*) was studied. By including the positive control infected by *H. annosum s.s.*, we were able to compare whether a single protein could induce similar defense responses as the pathogen. The results showed that relative to the negative control most of the selected marker genes, except for those under the control of the SA-mediated signaling pathway, were significantly upregulated in the seedlings treated with HaCPL2 and *H. annosum s.s.*. Exceptions were *JAZ1* and *ERF1*, whose expressions in the protein treatment were not significantly higher than the negative control (III, Fig. 6).

Many small cysteine-rich proteins have been identified as apoplastic effectors, *e.g. Phytophthora* elictins, *Cladosporium fulvum Avr2*, *Avr4*, and *Avr9*, *Rhynchosporium secalis nip1*, and *Stagonospora nodorum SnTox1* (Vandenackerveken *et al.*, 1992; Rohe *et al.*, 1995; Sasabe *et al.*, 2000; van Esse *et al.*, 2007; van Esse *et al.*, 2008; Liu *et al.*, 2012). Responses to both PTI and ETI include rapid influx of calcium ions, oxidative burst, activation of MAPKs, changes in protein phosphorylation, hormone biosynthesis, phytoalexin biosynthesis, reinforcement of cell walls at the site of attempted infection, receptor endocytosis, and transcriptional reprogramming (Schwessinger and Zipfel, 2008; Segonzac and Zipfel, 2011). During infection not only changes in secondary metabolism but also in primary metabolism are promoted to activate defense responses, with reduced growth and abnormal development in many cases (Berger *et al.*, 2007). Both early (transcriptional activation) and late (growth inhibition) responses were observed in the host plant after treatment with HaCPL2 and infection with *H. annosum s.s.*. The similar responses induced by HaCPL2 and the pathogen in Scots pine seedlings indicate that the host might perceive the presence of HaCPL2 as a signal of pathogen invasion and subsequently activate defense machinery.

The downstream responses to both PTI and ETI are controlled by signaling pathways mediated by salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Bari and Jones, 2009; Robert-Seilaniantz *et al.*, 2011). It has been established that JA- and ET-dependent responses are involved in plant defenses against necrotrophic pathogens and insect damage, while SA-dependent defenses are often triggered by biotrophic pathogens (Spoel *et al.*, 2007). In *N. tabacum*, the upregulation of SA-dependent marker genes *PR1a*, *PR2a*, and *PR10* is congruent with the activation of SA-mediated signaling pathway after treatment with HaCPL2 (Jwa *et al*., 2001; Jia *et al*., 2013; Kim and Hwang, 2014). *PAL* accumulation supports this assumption since the corresponding gene product acts as a positive regulator of SA-dependent defense signaling to combat microbial pathogens (Kim and Hwang, 2014). These results agree with the observations that infiltration of BcSpl1 from *B. cinerea* leads to the accumulation of SA in tobacco leaves (Frias *et al*., 2013). Moreover, *PR10* is a marker gene for HR-induced cell death, which supports our *in vivo* observations (Choi *et al*., 2012). The upregulation of *LOX* (*Lipoxygenase*) and the downregulation of *COI1* (*Coronatine-insensitive 1*) suggest that JA-dependent defense gene expression might be promoted by HaCPL2. COI1 contributes to the JA-mediated signaling pathway by promoting hormone-dependent ubiquitylation and degradation of transcriptional repressor JAZ (jasmonate zim domain) proteins (Schaller and Stintzi, 2009; Sheard *et al*., 2010). Therefore, we hypothesize that JAZ proteins are accumulated, acting as transcriptional repressors by interacting with MYC2 and promoting the defense-related gene expression (ERF branch of JA signaling) over wounding-related one (MYC branch of JA signaling) (Lorenzo *et al*., 2004; Fernandez-Calvo *et al*., 2011). The upregulation of *PAL* (*Phenylalanine ammonia lyase*) is in agreement with the accumulation of phytoalexins and other defensive secondary metabolites (Vogt, 2010). The increased mRNA transcripts of *PAD3* (*Phytoalexin-deficient* 3), a gene encoding for a key enzyme in camalexin biosynthesis, were also consistent with the activation of phytoalexin production (Zhou *et al*., 1999). In Scots pine seedlings, the JA-dependent marker genes *JAZ1* and *OPR* (*12-Oxophytodienoate reductase*) were significantly induced after treatment of HaCPL2 and *H. annosum s.s.*, suggesting the activation of JA-mediated signaling pathway (Schaller and Stintzi, 2009; Sheard *et al*., 2010). Additionally, the induction of *ACS* and *ERF1* suggests that JA and ET act concomitantly to control defense responses through the ERF branch of the JA/ET synergistic pathway. This branch of transcriptional regulation is known to be preferentially induced by necrotrophic pathogens (Lorenzo *et al*., 2004). Furthermore, *TPS* (*Terpene synthase*), *PME* (*Pectin methylesterases*), and *LAC* (*Laccase*), encoding for enzymes implicated in biosynthesis of terpenes, cell wall modification, or lignification, were induced (Micheli, 2001; Tholl, 2006; Berthet *et al*., 2012). *PAL1*, *NPR1* (*Natriuretic peptide receptor 1*, a positive regulator of SA signal transduction), or *LURP1* (*Late upregulation in response to Hpa 1*, required for full basal defenses against pathogens) was not differentially expressed; thus, no evidence emerged for induction of SA-dependent defenses or the phenylpropanoid pathway (Asai *et al*., 2000; Knoth and Eulgem, 2008). In *Arabidopsis thaliana*, cerato-platanin from *C. platani* has been found to induce camalexin biosynthesis and defenses mediated by SA and ET (Baccelli *et al*., 2014b). Nonetheless, the results in Scots pine are congruent with the induction of JA/ET-mediated signaling pathways after infection with necrotrophic pathogens (Zhu *et al*., 2011).

4.3.5. Conclusions for Study III

As one of the most economically important forest pathogens, *H. annosum s.s.* represents a well-characterized but complex lifestyle. The ability to switch the lifestyle between saprotrophic and necrotrophic phases requires the pathogen to be able to kill living tissues or cause necrotic cell death so that it can feed on the dead tissue. For the *H. annosum s.s.–Pinus sylvestris* pathosystem, we were able to identify a single protein that promotes plant defense responses, induces necrosis, and contributes to virulence. This has shed light on the potential role of cerato-platanins in the pathogenesis of *H. annosum s.s.* and provides a future target for disease control.

5. SUMMARY AND CONCLUSIONS

In this study, we performed a transcriptomic analysis of *H. annosum s.s.* under various abiotic stresses and during saprotrophic growth on different pine wood compartments. The results have elucidated the regulatory mechanisms for overcoming these unfavorable conditions and in early establishment on pine wood materials in nature. Furthermore, with the advantage of the published fungal genomes, we were able to carry out a genome-wide bioinformatic analysis of the cerato-platanin family in Dikarya. Results indicated that the ancestor of the Dikarya possessed multiple copies of cerato-platanins, which sorted differently in Ascomycota and Basidiomycota. Moreover, gene loss and gene expansion were also observed during the evolution of this family. Although no correlation was found between homolog number and fungal nutritional mode, we were able to demonstrate that HaCPL2 could reproduce all or at least part of the disease symptoms produced by the intact pathogen. This suggests that HaCPL2 contributes to the virulence of *H. annosum s.s,* which furthers our understanding of the pathogenesis of the pathogen and provides a future target for disease control.

6. FUTURE PERSPECTIVES

Although *H. irregulare* is a closely related species, elucidation of the genome sequence of *H. annosum s.s.* would improve the accuracy of the microarray data. Since the cost for sequencing has become reasonable, next-generation sequencing including RNA-seq would be a good alternative way for transcriptomic analysis, which can be combined with other high throughput approaches like proteomics and metabolomics.

Due to the low efficiency of constructing a knockout mutant of *H. annosum s.s.*, we decided to study the function of HaCPL2 through a recombinant protein produced by *P. pastoris*. Although HaCPL2 was able to induce cell death and defense responses in host and non-host plants, the mechanisms for how the plants perceive the protein remain unclear. Necrotrophic effectors have been reported to rely on a series of positive fungal effector–host dominant sensitivity receptor interactions to result in necrosis and disease. It would be interesting to identify the receptors in *P. sylvestris* for HaCPL2 recognition and to determine whether recognition can be separated from the intrinsic activity of the protein. Moreover, in this study, a mixture of different forms of HaCPL2 was used for bioassay on plants. This might be improved by size exclusion chromatography followed by bioassay of their activity separately if the various forms of HaCPL2 are stable. This would reveal the active forms of HaCPL2. Finally, to understand the role of a gene in a host–pathogen interaction, it is essential to perform a virulence analysis of gene knockout mutants followed by gene complementation experiments. This would provide direct evidence of involvement in pathogenesis or contribution to virulence and confirm their biological roles in the pathosystem. Therefore, development of a functional and efficient DNA transformation system for *H. annosum s.s.* merits future research.

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Hongxin

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